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bei RESOURCES
SUPPORTING INFECTIOUS DISEASE RESEARCH

Sequence Determination of Hybridoma Antibody Transcripts Targeting Virulent or Immunogenic Factors of *Mycobacterium tuberculosis*

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Hui-Chen (Jane) Chang Foreman, PhD, Andrew Frank, MS, Timothy T. Stedman, PhD
BEI Resources, Manassas VA 20110; ATCC, Manassas, VA 20110

Abstract

Mycobacterium tuberculosis (Mtb), a causative agent for tuberculosis (TB), remains one of the most challenging pathogens to control. Mtb infects nearly a quarter of the world's population and sinisterly synergizes with HIV to claim the lives of around 1/3 of all AIDS patients. While the WHO has considered this epidemic a global emergency since 1994, TB control has been hampered by lack of protective vaccines and a rapid, effective diagnostic tool. Only in recent years has TB antibody development offered new potential to control TB infections. The humoral antibody functions of TB immunity have been discovered and distinguishable antibody patterns in active/chronic stages of TB have been uncovered. Thus, knowledge of the paratope sequences of Mtb antibodies enables engineering diagnostic and therapeutic tools. Currently, few validated TB antibody sequences are available. Here, we identified the sequence of functional variable immunoglobulins (IgVs) expressed in 14 hybridomas encoding antibodies to Mtb targets with potential therapeutic/diagnostic value: (1) Mpt64, a mycobacterial diagnostic peptide; (2) Ag85 complex, the most immunogenic Mtb protein to date; (3) the Mtb bacterial surface components (A) glycolipid LAM, (B) lipoprotein LprG, and (C) HBHA, an epithelial cell adhesion factor; (4) the Mtb enzymes (A) Superoxide Dismutase SodA and (B) Catalase KatG, crucial for Mtb survival within the hostile macrophage phagosome; (5) the Mtb regulatory factors PhoS1/PstS1, factors within the ABC transporter system; and (6) the Mtb Heat Shock Proteins HspX, DnaK, and GroES. We isolated the paratope-determining CDR 1-3 regions of the heavy and light chains of these IgVs using a 5'RACE-PCR amplification from the cDNA of each hybridoma via an isotype (gene)-specific primer (GSP) for each of the light chains of Igκ/Igλ_1, 2, and 3 and heavy chains of IgG1, IgG2a, IgG3, and IgM. Using an Illumina® NGS-MiSeq™, 2X150bp, pair-wise sequencing platform, we generated 28 IgV libraries. Most libraries contained sufficient reads and coverage for *de novo* assembly of Ig chains via a bioinformatics algorithm workflow for analysis of Ig sequences. Thirty-three (33) putative TB IgV sequences were identified. Validation of their antigen-binding capability via recombinant antibody techniques is in progress.

Methods

- Mtb hybridomas from BEI Resources (Table 1) were cultured in DMEM medium (ATCC® 30-2002™) supplemented with 10% FBS (ATCC® SCRR-302020™).
- Antibody Isotypes were determined using mouse antibody kits (Pierce™ Rapid Isotyping kit plus Kappa and Lambda, Thermo Scientific 26179; IsoStrip™ mouse monoclonal antibody isotyping kit (Roche 11493027001)
- Total RNAs were extracted. The integrity of RNA was evaluated by spectrometry and gel electrophoresis.
- Complementary DNA (cDNA) were reverse transcribed. 5'-RACE PCR was performed with the gene (isotype) specific primers (GSP) to the highly conserved constant regions of the antibody.
- Variable heavy and light chain sequences from the 14 hybridomas were determined by either Sanger or by Next Generation Sequencing (NGS), de novo assembled via an established bioinformatics algorithm workflow.

Table 1. List and Features of BEI Resources *M. tuberculosis* Hybridomas

BEI Resources Item No.	Clone #	Isotype	Antigen	Target gene	PMID
NRC-13806	Clone a-Rv1411c	IgG1κ	LprG/P27	Rv1411c	21364279 23562367
NRC-13810	CS-18	IgG1κ	SodA	Rv03846	24586151
NRC-49679	clone A	IgG1κ	DnaK	Rv0350	n/a
NRC-49680	clone A	IgMκ	KatG	Rv1908c	n/a
NRC-2893 (NRC-13811)	CS-35	IgG3κ	LAM	n/a	21820887
NRC-2894	IT-3 (SA-12)	IgG2aκ	GroES	Rv3418c	n/a
NRC-2895	CS-49	IgG1λ1	HspX	Rv2031c	n/a
NRC-2897	CS-90	IgMκ	Ag85 complex	Rv3804c Rv1886c Rv0129c	23562367
NRC-50100	Anti-DnaK, Clone B	IgG1κ	DnaK	Rv0350	n/a
NRC-50101	Anti-KatG, Clone B	IgMκ	KatG	Rv1908c	n/a
NRC-50703	Anti-Mpt64, Clone A	IgG1κ	Mpt64	Rv1980c	n/a
NRC-50704	Anti-Mpt64, Clone B	IgG1κ	Mpt64	Rv1980c	n/a
NRC-2914	a-HBHA	IgG2aκ	HBHA	Rv0475	22363768
NRC-2410	IT-15 (TB72)	IgG1κ	PhoS1/PstS1	Rv0934	n/a

Results

(1) Extraction of mRNA

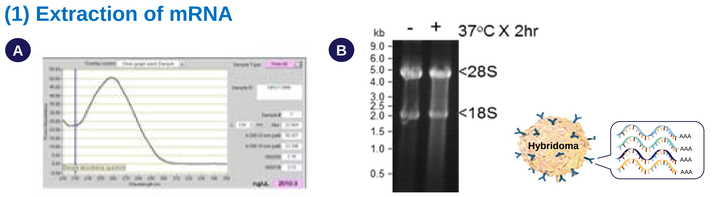


Figure 1. RNA extracts from the 14 clones yielded high quality RNA with minimal RNase contamination. (A) RNA purity for NRC-13806, representative of the 14 Hybridoma clones using a NanoDrop™ 2000 Spectrophotometer. The OD_{260}/OD_{280} ratio is greater than 1.9, indicating high purity. (B) NRC-13806 RNA was resolved using 1.25% formaldehyde gel electrophoresis with (+) or without (-) incubation at 37°C for 2 hours to assess RNase contamination. The ratio of ribosomal 28S/18S remains constant, indicating minimal RNase.

(2) RACE- Ig PCR

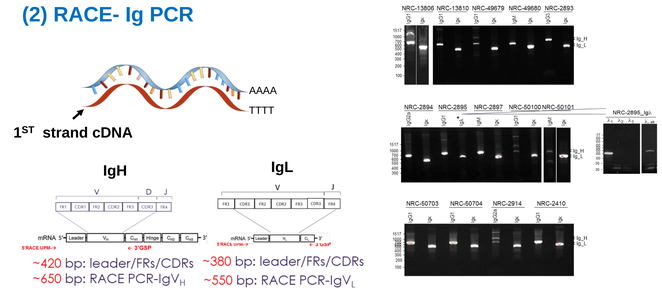


Figure 2. RACE PCR immunoglobulin heavy (IgH) and light (IgL) chain products for each of the 14 Hybridoma cell lines. RACE PCR used 5' Universal primers (5'-UPM) and Ig specific Gene Specific Primers (GSP) to amplify variable regions of Ig fragments.

(3) Sequencing Ig Fragments

TOPO/Sanger Clonal Sequencing

WORKLOADS

28 PCR-IgVs
- Cloning to a TOPO-vector
- Screening 28 X (7-10) clones (>200 clones)
- Sanger Sequencing

Illumina® NGS

28 libraries via NGS/Illumina® MiSeq™ platforms
- V2: 2X150 bp

MinION™/GridION™ Nanopore® Sequencing

GridION™ X5 Sequencer platform
- Pioneering

NGS/Bioinformatics Analysis

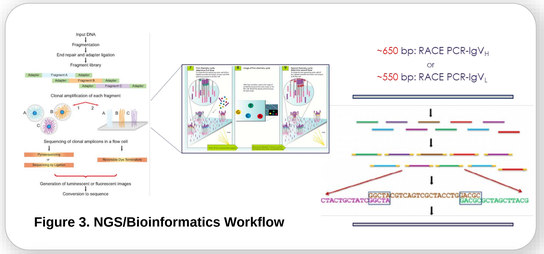
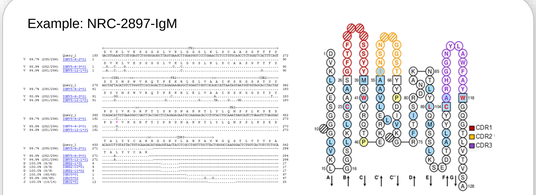
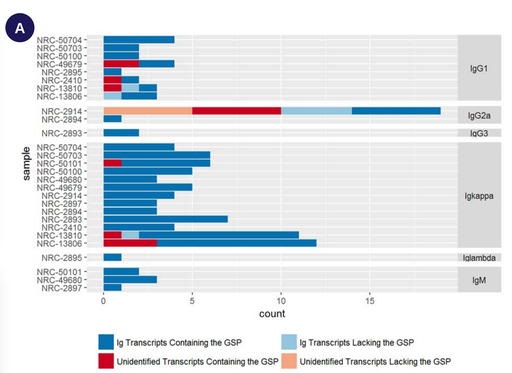


Figure 3. NGS/Bioinformatics Workflow



KABAT algorithm
https://www.ncbi.nlm.nih.gov/igblast/igblast.cgi

IMGT algorithm
http://www.imgt.org/IMGT_vquest/vquest



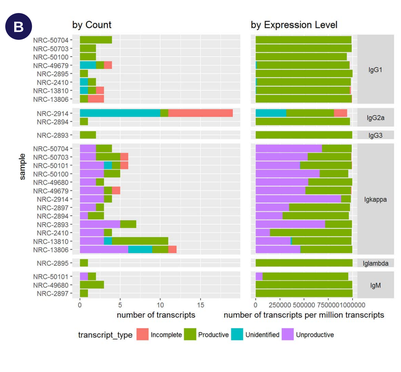


Figure 4. Profiles of Contig types among the 14 hybridomas. (A) Summary of hybridoma sequence contig assemblies identified using isotype specific GSPs. Graph was generated using grggplot2 (https://ggplot2.tidyverse.org/). (B) The Trinity *de novo* assembled Ig contigs were grouped/quantitated based on sequence type (by count) or abundance (by expression level):

- Unique Productive - contigs without STOP codons
- Unproductive - contigs with one or more STOP codons in the CDRs
- Incomplete - only 1 or 2 CDRs identified
- Unidentified - no CDRs identified.

Summary and Perspectives

- The established & validated NGS/Bioinformatics platform for Igs successfully identified IgV sequences for all 14 *M. tuberculosis* hybridomas.
- Five hybridomas, encompassing all isotypes of this project, were assessed for sequence validation by GenScript®
- ATCC analysis was consistent, but an additional IgV sequence was found in one clone, suggesting equal or better performance for identifying Ig sequences.
- Validation of IgV epitope binding via an antibody recombinant system is underway.

Acknowledgements

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